EXHIBIT A

Review

Markus F. Templin
Dieter Stoll
Jochen M. Schwenk
Oliver Pötz
Stefan Kramer
Thomas O. Joos

NMI Natural and
Medical Sciences Institute
at the University of Tuebingen,
Reutlingen, Germany

Protein microarrays: Promising tools for proteomic research

Miniaturized and parallelized ligand binding assays are of great interest in postgenomic research because microarray technology allows the simultaneous determination of a large number of parameters from a minute amount of sample within a single experiment. Assay systems based on this technology are used for the identification and quantification of proteins as well as for the study of protein interactions. Protein affinity assays have been implemented that allow the analysis of interactions between proteins with other proteins, peptides, low molecular weight compounds, oligosaccharides or DNA. Microarray technology is an emerging technology used in global analytical approaches and has a considerable impact on proteomic research.

Keywords: Antibody array / Biochip / Cell array / Microarray / Protein array / Review / Reverse screening
PRO 0600

Contents

1 Introduction

The fundamental principles of miniaturized microspot ligand-binding assays were already described in the eighties by Roger Ekins in his "ambient analyte theory" [1, 2] which suggested that miniaturized ligand-binding assays were able to achieve superior sensitivity. The small number of capture molecules in a spot on a microarray allows the conditions to be met that are described by the ambient analyte theory. Consequently, only a small number of analytes or target proteins can be captured on a spot. The actual concentration of the analyte molecules in the sample does not change significantly, even in the case of targets of low concentration and high affinity binding reactions. Ligand-binding assays allow the determination of the concentration because the amount of the target or analyte captured from the solution directly reflects its concentration in the solution. In addition, the measurement of the concentration under ambient analyte conditions renders the system independent of the actual sample volume. High sensitivity is achieved because the binding reaction occurs at the site of the highest possible analyte concentration. As the capture-molecule-analyte complex is only concentrated in a small area of a microspot, this results in a much higher signal compared to a macrospot. Therefore, highest signal intensities *per* area and optimal signal-to-noise ratios can be achieved under ambient analyte conditions. Thus, femtomolar concentrations could be detected in the investigation of analytes such as thyroid stimulating hormone or Hepatitis B surface antigen [3]. Parallelization and high sensitivity of microarray technology have an enormous potential for diagnostic applications. However, it is not the diagnostic field which has been the driving force for the rapid development of microarray technology but the urgent demand within the field of genomics for global analytical tools [4]. The possibility to determine thousands of different pa-

Correspondence: Dr. Thomas O. Joos, NMI Natural and Medical Sciences Institute at the University of Tuebingen, Markwiesenstr. 55, D-72770 Reutlingen, Germany
E-mail: joos@nmi.de
Fax: +49-7121-51530-16

rameters in a single experiment perfectly suited the needs of genomic approaches in biology. Today, DNA microarray technology is a robust and reliable method and allows a global view into the transcriptome of a living cell or organism on the basis of a single experiment [5–7]. However, the cellular functions of a living cell are mediated by the products that are encoded by the genes, namely the proteins. Therefore, the accurate description of biological processes requires in-depth knowledge of protein expression and even more so, a knowledge of the functional state of proteins. There is no close correlation between mRNA expression and protein expression [8–10]. In general, the function of a protein is not just regulated by its presence or absence but by post-translational modifications like phosphorylation and dephosphorylation, by compartmentalization and/or by specific interactions between proteins. Therefore, information that goes beyond the protein expression level is required for the analysis of complex biological systems. Once again, microarray technology has the great potential to provide us with powerful tools to identify and quantify proteins and to study their function in global perspectives [11, 12].

2 Protein microarray assays

Protein microarray assays allow the identification and quantification of a large number of target proteins from a minute amount of a sample within a single experiment. In addition, protein microarrays are powerful tools that can be used for the analysis of interactions between proteins with other proteins, peptides, low molecular weight compounds, oligosaccharides or DNA (Fig. 1, A–L) [11, 12]. Technologies established for DNA-chip applications have been adapted to cater for the needs of protein microarray-based research. There are hundreds of companies and technology providers on the market offering products such as microarray surfaces, arrayers and detection systems for the fast growing DNA and protein microarray community (http://www.biochipnet.de). However, the availability of highly specific capture molecules is the main difference between the DNA and the protein world. Today, the lack of specific capture molecules is the main factor which still limits the broader use of protein microarray technology.

DNA is a very uniform and stable molecule which binds its complementary targets by means of the well defined base-pairing principle. Due to the complementary nature of the DNA molecule, capture sequences can easily be predicted from the primary DNA sequence of the target molecules. Efficient oligonucleotide synthesis or PCR-based approaches enable the fast and economic generation of a large variety of DNA capture agents. This is different for proteins. There is no one-by-one interaction as observed for DNA base pairing. Proteins exhibit very diverse and individual tertiary molecular structures. Their binding interaction takes place by different means such as electrostatic forces, hydrogen bonds and/or weak hydrophobic Van der Waals interactions. In addition, individual proteins can even interact with different binding partners at the same time and in a synergistic way. At present, there is no way which would allow the prediction of high affinity protein capture molecules only on the basis of their primary amino acid sequence. Steady or dynamic post-translational modifications like glycosylation, phosphorylation, and acetylation must also be taken into consideration (Table 1).

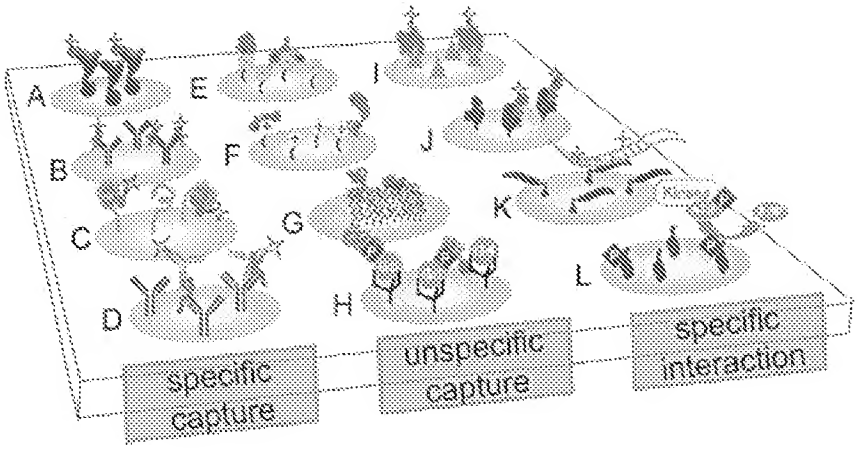


Figure 1. Types of protein interaction and protein capture microarrays. Specific protein capture on microarrays by affibodies (A), antibodies (B), aptamers (C) or antibody sandwich formation (D). Unspecific capture is based on electrostatic (E, F), van der Waals – hydrophobic (G) or metal – chelate (H) interactions. Captured proteins have to be identified using mass spectrometry (*e.g.* SELDI). Specific interaction microarrays have been described for receptor-ligand (I), protein-protein (J), protein-DNA (K) and enzyme–substrate interactions.

Table 1. Properties of DNA and proteins with respect to their application in microarray technology

Properties	DNA	Protein
Structure	Uniform Hydrophilic acidic backbone Stable	Individual types Hydrophobic and/or hydrophilic domains Fragile
Functional state	Denatured, no loss of activity → can be stored dry	3-D structure important for activity, → avoid denaturation
Interaction sites	1 by 1 interaction	Multiple active interaction sites
Interaction affinity	High	Dependent on individual protein: very low to high
Interaction specificity	High	Dependent on individual protein: very low to high
Activity prediction	Well defined Based on primary nucleotide sequence	Not possible yet. Efforts are undertaken to predict models that are based on sequence homologies, structure, *etc.*
Amplification	Established (PCR)	Not available yet

2.1 Protein identification and quantification

A large number of highly specific capture molecules which exhibit high affinity to their target molecules are a prerequisite for the establishment of protein microarrays used for the identification and quantification of target proteins. Different types of possible capture agents are summarized in Table 2.

Antibodies are highly specific targeting agents and valuable tools for *in vitro* and *in vivo* diagnostic applications. A major improvement of antibody generation could be achieved with the application of monoclonal antibodies which represent a virtually unlimited source of uniform, pure and highly specific binding molecules [15]. However, antibodies, be they polyclonal or monoclonal, have some disadvantages in terms of generation, cost and overall application. The continuous culture of hybridoma cells, which leads to monoclonal antibodies (mAb), offers the potential of an unlimited supply of reagent when compared with the rather limited supply of polyclonal antibodies. The virtually unlimited supply enables the standardization of both reagent and assay technique. However, due to the labor-intensive nature of mAb production,

Table 2.

Capture molecules	Source	Technique	Reference
mAb	Mouse	Hybridoma	Reviewed in [13–15]
Polyclonal sera	Rabbit, mouse, goat, chicken		[16, 17]
scFv/Fab	Antibody libraries	Phage display	[18–25]
Diabodies		*In vitro* evolution	
Affinity binding agents scaffolds	Recombinant Fibronectin structures Ankyrin repeats	*In vitro* evolution	[26–28]
Affibodies	Microorganism	Heterologous expression	[29, 30]
Aptamers (DNA/RNA/peptide)	Library	SELEX/mRNA display *In vitro* evolution	[31–34]

further efforts have been undertaken to develop alternative technologies. The most promising approaches in this field involve phage display techniques [20, 22] combined with highly diverse synthetic libraries (up to 10^{11} independent clones). This enables the fast and efficient production of ultra diverse protein molecules and leads to the selection of binder molecules which can be directed against nearly any target within weeks. Many of these antibodies exhibit binding affinities with a K_D in the nM range. Maturation strategies allow the improvement of affinities to a dissociation constant K_D in the pM range [18, 36].

Other approaches to generate specific binding molecules from synthetic libraries led to the discovery of aptamers. These are short polymers (ten to several hundred building blocks) like oligonucleotides, peptides or similar molecules peptide nucleic acids (PNAs) [37] or locked nucleic acids (LNAs) [38]). Short peptides [39] with the ability to recognize virtually any type of target molecule with high affinity and specificity have been described. Aptamer oligonucleotides can be generated with an *in vitro* evolution process called SELEX (Systematic Evolution of Ligands by EXponential enrichment). Although their molecular properties are different from those of antibodies, they mimic the molecular recognition properties of antibodies. Compared with the highly skilled antibody technologies, aptamer research is still in its infancy but progressing at a fast pace. Different scaffolds have also been used for the recombinant production of capture molecules. Examples of such molecules include the receptins, trinectins, ankyrins, and anticalins [29, 30, 40–42]. Scaffolds obtained can also be subjected to affinity maturation procedures and multimerization in order to generate high affinity binders.

2.1.1 Miniaturized and parallelized immunoassays

Miniaturized and parallelized sandwich immunoassays are of general interest for all proteomic and diagnostic approaches in which several parameters have to be determined simultaneously from a limited amount of sample material. Besides microarray-based systems, which are perfectly suited to screen for a large number of target proteins, bead-based assays are a very interesting alternative to the planar microarrays, especially when the number of parameters of interest is comparably low (Fig. 2). Bead-based assay systems employ different color-coded microspheres as the solid support for the capture molecules and a flow cytometer which is able to identify each individual type of bead and to quantify the amount of captured targets on each individual bead. Sensitivity, reliability and accuracy are similar to those

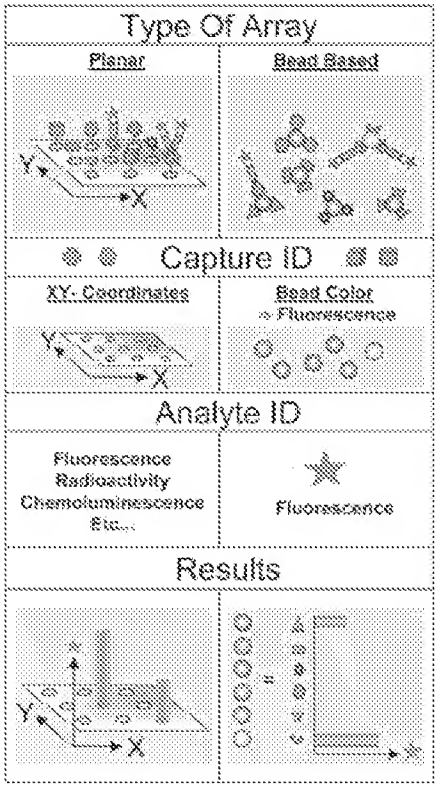


Figure 2. Types of microarrays. Planar microarrays and bead-based arrays represent two different microarray technologies that can be employed for multiplexed ligand-binding assays. Planar microarrays can be generated with hundreds and thousands of different capture spots. Multiplexing in bead-based arrays is limited to the number of distinguishable beads. Separation of beads is performed *via* color coding (*e.g.* Luminex). Detection on planar arrays is performed using different detection principles, like chemoluminescence, radioactivity, mass spectrometry or fluorescence. Fluorescence is mainly used for the readout of bound analytes in bead-based microarray assays.

observed with standard microtiter ELISA procedures. Color-coded microspheres can be used to perform up to a hundred different assay types simultaneously (LabMAP system, Laboratory Multiple Analyte Profiling; Luminex, Austin, TX, USA) [43–47]. For example, microsphere-based systems have been used to determine the concentration of cytokines or antibodies in biological samples such as patient serum or cell culture supernatant [44, 45, 48–51].

Sandwich immunoassays have also been adapted to a microarray format [52–57]. Huang *et al.* [53] performed highly specific and sensitive protein microarrays and were able to identify and quantify 24 different cytokines

from conditioned media and patient sera. Schweitzer *et al.* [57] have described the most complex multiplexed sandwich immunoassay so far which allowed the quantification of 75 different cytokines. However, it was not possible to perform a 75-plex sandwich immunoassay within a single microarray due to cross-reactivity of some of the detection antibodies with immobilized capture antibodies or due to cross-reactivity with the nonspecific analytes. To separate the different cross-reacting antibodies, two sets of multiplexed sandwich immunoassays were generated containing 38 or 37 distinct features in a single microarray, respectively. These microarrays were used to study the expression of target protein present in stimulated and nonstimulated human dentritic cells. The highly sensitive isothermal rolling circle amplification method was used for detection purposes and different cytokines could be detected in the femtomolar concentration range. In principle, it should be feasible to detect single binding events on microspots using this amplification method [57, 58].

Miniaturized and multiplexed immunoassays offer an appropriate solution for applications in which several parameters of a single sample have to be analyzed in parallel. This is important in autoimmune diagnostics when patient sera are to be screened for the presence or absence of a large number of different types of autoantibodies [59, 60]. Autoantigens used as diagnostic markers for autoimmune diseases such as systemic rheumatic diseases were immobilized in a microarray and different types of autoantibodies could be accurately determined from less than one microliter patient serum. This reflects the enormous potential of protein microarrays employed to study the humoral response against a large number of antigens. Microarrays have also been successfully demonstrated to be able to reveal the presence of specific IgG and IgM antibodies directed against parasitic and viral antigens such as *Toxoplasma gondii*, *Cytomegalo*, *Herpes simplex* and *Rubella virus* [61]. In addition, the microarrays included internal calibration curves for IgG and IgM in order to quantify the immune response of the patients. The analytical sensitivity of these assays was similar to those obtained with standard ELISA technology [61].

The next critical step will involve the validation of these assay systems and to define the diagnostic needs. Immunoassays used in clinical diagnostics should allow for automation, which leads to increased assay performance and reduced assay time. Any new assay format for diagnostic applications has to compete with the currently used highly robust technology. Automated immunoassay analyzers are well established in the diagnostic market. New instruments in combination with a new multiplexed assay formats will require a huge investment. This is particularly true if the number of parameters to be determined is low. In these cases, the diagnostic industry will no doubt hesitate to switch to a new format. As long as only a few additional parameters have to be analyzed from the same sample, it is much more economic to increase the throughput of the currently used diagnostic analyzers. However, allergy diagnostics might find microarrays quite a suitable format as a sufficiently high number of parameters must be determined. This will render microarrays or bead-based systems a very interesting technological alternative [62–65]. In general, allergies are being diagnosed by provocation testing and employing IgE serology which makes use of allergen extracts or complex mixtures of different potential allergens. The identification of the disease-causing allergenic molecules is a costly and time-consuming procedure. Miniaturized and multiplexed ligand-binding assays can circumvent most of the current limitations. Hiller *et al.* [64] used 78 recombinant and 16 purified allergen molecules to generate a microarray. The individual IgE reactivity towards the microarrayed allergenic components reflected the clinical sensitivity of the individual patients to the different allergens. The sensitivity of the assay could mainly be achieved by immobilizing purified allergens. Signal enhancement procedures such as the rolling circle or tyramide amplification systems are required if complex allergen extracts are to be immobilized in individual microspots [58, 62–66].

Planar microarrays or bead-based systems are very well suited for multiplexed immunoassays (Fig. 2). Accurate quantification and control of assay performance and reproducibility can be achieved by including several positive and negative controls and/or internal calibration standards. Thus, multiplexed miniaturized sandwich immunoassays have the enormous potential of becoming robust and reliable proteomic and diagnostic tools.

2.1.2 Protein microarrays for proteomics

The potential of array-based proteomic approaches has recently been discussed in several reviews [67–70]. Protein microarray technology can be used to determine the amount of a large number of target proteins. More importantly, it can also be used to detect changes in co- and post-translational modifications. Capture molecules, *e.g.* highly specific and selective antibodies, are immobilized in a microarray format in the same way as in DNA microarrays. Cell extracts that are quantitatively labeled with fluorescent markers are incubated on the array. Subsequently, bound proteins can be detected with the instruments that are used for conventional DNA microarray technology.

In analogy with DNA chip technology, Brian Haab and colleagues [71] adapted the dual-color labeling procedure to antibody-antigen microarrays (Fig. 3). To prove their concept of differential protein analysis, Haab *et al.* used a set of 110 defined antigen and antibody pairs to create microarray-based immunoassays. Antibodies or antigens were immobilized on the surface and the corresponding targets were fluorescently labeled in complex solutions such as serum, for example. One sample that was spiked with antigens was labeled with Cy5; a second sample containing a different concentration of the antigens was labeled with Cy3-fluorophores. Subsequently, the two samples were mixed and simultaneously incubated on the same microarray. The dual-color detection system was able to reveal the different concentrations of the captured targets. Depending on the individual affinities of the antibodies, it was possible to detect the antigens at picomolar concentrations.

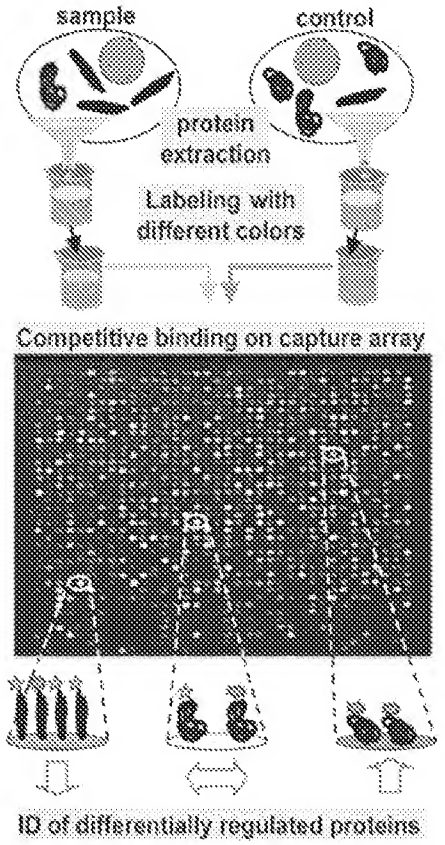


Figure 3. Principle of differential capture-protein microarray assays. Proteins from controls and samples are isolated, labeled with two different colors (fluorophores), mixed and incubated on the same protein-capture microarray. Two-color detection of bound proteins directly reveals the differences in protein expression between control and sample.

Sreekumar *et al.* [72, 73] used this two-color labeling approach to study changes in the protein expression level of LoVo colon carcinoma cells after an ionizing radiation treatment. Lysates of treated and nontreated cells were labeled with Cy3 and Cy5, mixed and allowed to bind to an antibody microarray containing 146 distinct antibodies directed against proteins involved in apoptosis, stress response, and the cell cycle. The comparative analysis revealed the up-regulation of eleven distinct proteins and the down-regulation of a single protein. Until then, only five of the eleven up-regulated proteins had been known to be involved in a response to the ionizing radiation treatment. Knecevic *et al.* [74] were able to show cancer-specific alterations in the expression of proteins using an antibody microarray containing 368 antibodies. Cancerous epithelium in the oral cavity, normal cells, and cells surrounding these tissues were microdissected, lysed, biotinylated and incubated on the arrays. Bound proteins were detected using a biotin-based signal amplification. Eleven distinct proteins were identified which altered their expression level in correlation to tumor progression. Both approaches nicely reflect the power of antibody microarrays when it comes to studying changes in protein expression in a single experiment. Results obtained with antibody microarrays have to be verified and confirmed with standard methods, since one has to be aware that some antibodies exhibit a strong cross-reactivity. In addition, proteins are often assembled in multiprotein complexes. A strong signal on a microspot can therefore result not only from a large amount of target molecules but also from the capture of a huge labeled complex.

Antibody microarrays which consist of several hundred mAbs are already commercially available (BD Biosciences Clontech, www.bdbiosciences.com; Hypromatrix, www.hypromatrix.com). In addition, enormous efforts have been undertaken to produce a large number of highly selective capture molecules for use in antibody arrays for global protein profiling. Protein microarrays certainly have the potential to extend molecular analysis beyond the limitations of DNA chips.

An alternative antibody microarray-based approach avoids the possibility of large protein complexes being captured [75]. This peptidomics approach starts by enzymatically cleaving the analyte proteins into peptides which are afterwards captured on arrays of peptide-specific antibodies. The bound peptides are identified by MALDI-MS which circumvents some of the drawbacks of direct protein capture assays such as protein solubilization, protein complex formation, *etc.* However, it still has to show its benefits for application in large-scale protein profiling.

2.2 Protein interaction analysis

Protein-microarray based interaction analysis has been described for the analysis of protein-protein, enzyme-substrate, protein-DNA, protein-oligosaccharide and protein-drug interactions. Low and high density protein arrays were used to investigate the binding of DNA, RNA, small chemical ligands and proteins. Enzyme-substrate assays were performed for restriction enzymes, phosphatases, peroxidases and phosphokinases and have the potential to provide functional data on a genome-wide scale. They are also required for the functional analysis of complex protein networks within biological systems.

2.2.1 Protein-protein interactions

Protein-protein interaction assays involve dot-blot filter arrays to screen for specific interactions of immobilized proteins with other proteins. The filters are loaded with highly purified and fully active recombinant proteins. This so-called universal protein array (UPA) system is a very effective method which allows screening of protein interactions at low cost. Ge, for example, was able to detect specific protein-protein interactions between a radioactively-labeled human p52 GST fusion protein and immobilized capture proteins such as nucleoline or a serine-argenine protein fraction isolated from HeLa cells [76]. In addition, interactions of DNA, RNA, or low molecular weight ligands with immobilized molecules were observed. In principle, the miniaturization of UPA arrays seems possible.

Recently, protein microarrays were used to analyze protein interaction using various protein domains. These included the SH3 and SH2 (Src homology 2 and 3), PDZ (a domain originally identified in PSD-95, DLG and ZO-1 proteins), PH (pleckstrin homology), and FHA (forkhead-associated) domains which contain short peptide motifs that serve as recognition modules for the assembly of multiprotein complexes [77]. The peptides were fused to glutathione-S-transferase and a protein-domain microarray was prepared. Proteins like Sam68 (Src-associated during mitosis 68) bound to their specific SH3 and WW domains.

Global protein interaction studies were performed with a yeast proteome chip. Zhu *et al.* [78] generated a yeast proteome chip from recombinant protein probes of 5800 open reading frames. To test for protein-protein interactions, the yeast proteome was probed with biotinylated calmodulin. Many known CamKinases and calcineurins were identified. In addition, 33 new potential binding partners of calmodulin with a potential binding motif were found. With these studies, Zhu *et al.* were, for the first time, able to present a genome-wide analysis of proteins that interacted with phospholipids. Six different types of liposomes were used to identify a total of 150 different protein targets including integral membrane proteins and peripherally-associated proteins, for example. Many of the uncharacterized proteins were said to be membrane associated, which was taken as an indication that they preferentially bind specific phospholipids *in vivo*. This study clearly demonstrated the advantage of an approach using a proteome chip. An entire proteome can be prepared and directly screened *in vitro* for a wide variety of activities including protein-drug interactions and protein-lipid interactions, which might not be accessible by other approaches. The generation of protein arrays of several 10 000 proteins for global proteome analysis in humans and other eukaryotes is feasible using similar procedures.

2.2.2 Enzyme-substrate assays

Enzyme-substrate arrays have been described for different kinds of enzymes such as restriction enzymes, peroxidase, phosphatase and protein kinases [76, 79–85]. In a proof-of-concept experiment, MacBeath and Schreiber [82] immobilized three different kinase substrates onto a planar glass surface. Identical microarrays were individually incubated with one specific kinase together with radioactively labeled ATP. Each substrate was phosphorylated only by its specific kinase. In a more advanced approach, Zhu *et al.* [80] analyzed the activities of 119 of the 122 known or suspected protein kinases from *Saccheromyces cerevisiae* for 17 different substrates. They used microwell plates with substrates covalently linked to individual microwells. The overexpressed, purified kinases were subsequently incubated on these microwell arrays along with radioactively labeled ATP. After completion of the reaction, kinases and the non-incorporated radioactive ATP were washed away and the arrays analyzed for phosphorylated substrates by a phosphoimager. Novel activities of individual kinases could thus be identified. Sequence comparison of enzymes which phosphorylate tyrosine residues revealed that they often share common amino acid residues around their catalytic region.

Peptide arrays built from large sets of individual peptides or peptide libraries have been used to screen for unknown enzymatic activities or for the selection of antibodies [84, 85]. In an approach to quantify cathepsins and caspases, Winssinger *et al.* [92] combined a fluid phase assay with planar microarray technology. Seven enzyme-specific inhibitors were fluorescently labeled and coupled to specif-

ic PNA (peptide nucleic acids) tags. After incubation with treated and untreated cell lysates, unbound inhibitors were removed and the enzyme-inhibitor complexes were captured on a DNA chip containing the complementary DNA sequences in individual microspots. Signal intensity correlated with the amount of active cathepsins and caspases present in the differentially treated cell lines [92].

2.2.3 Protein-DNA interactions

Studies on DNA-protein interactions in a microarray format were performed by Bulyk *et al.* [79] who created microarrays of distinct dsDNA sequences. DNA cleavage did not occur when the dsDNA was enzymatically methylated prior to the incubation with the specific restriction enzymes. This example clearly illustrates that it is possible to modify dsDNA arrays biochemically. The same group described a DNA microarray-based method used to characterize sequence-specific DNA recognition by zinc-finger proteins [86]. In general, DNA-protein interaction assays have proven useful in the characterization and identification of DNA-binding proteins.

2.2.4 Carbohydrate-protein interactions

Carbohydrates are the key components of glycoproteins, glycolipids and proteoglycans. They are involved in recognition processes such as cell adhesion, migration and signaling. Protein-carbohydrate interactions are essential for many biological processes including normal tissue growth and repair, cell-cell adhesion and inflammation, cell growth, fertilization, viral replications, parasitic infection, as well as tumor-cell motility and progression. Alterations of glycosylation events are also involved in a number of diseases.

Recently, Fukui *et al.* [89] generated oligosaccharide microarrays in order to study carbohydrate-protein interactions. Oligosaccharides derived from glycoproteins, proteoglycans, glycolipids or synthetic oligosaccharides were linked to lipids to form neoglycolipids which were immobilized on membrane microarrays and incubated with proteins such as the leukocyte-endothelium adhesion molecules E- and L-selectin, the cytokine interferon-γ (IFN-γ) and the chemokine RANTES. All known target carbohydrates bound to these proteins. In addition, IFN-γ and RANTES do not only interact with oligosaccharides of chondroitin sulfates but also with other sulfated moieties that are present on natural killer and epithelial cells. These sequence-specific interactions could be involved in tissue targeting or IFN-γ and RANTES signaling. Carbohydrate microarrays offer an interesting possibility to identify additional binding motifs of target proteins.

Wang. *et al.* [90] used carbohydrate microarrays to analyze host-pathogen interactions. Forty-eight microbial polysaccharide glycans were printed onto a nitrocellulose membrane and incubated with human sera to identify antibodies directed against the different types of microbial polysaccharides. The assays revealed differences in the anticarbohydrate antibody repertoire of normal individuals in comparison to myeloma and lymphona patients. Such carbohydrate arrays could be an interesting approach to determine the kind of infection an individual has or had, and whether there is an increase in antibodies to a specific carbohydrate moiety.

2.2.5 Protein-small molecule interactions

Data from genomic or proteomic analyses can also be used in drug screening processes of the pharmaceutical industry. Microarrays of immobilized proteins and small organic compounds might be powerful tools for future high-throughput drug screening technologies. Receptor-ligand assays involve small organic molecules that are produced by combinatorial solid phase chemistry and immobilized in a microarray format. MacBeath and coworkers [91] placed single resin beads in 96-well plates; the organic molecules were chemically released from the beads, diluted, spotted and covalently attached to derivatized glass slides. These microarrays, produced by small molecule printing technology, were incubated with fluorescently labeled target proteins in order to identify new ligands. This technology enables parallel high-throughput screening for ligand-receptor interactions and only requires very small sample quantities. This could certainly help improve procedures aimed at screening for active substances employed by the pharmaceutical industry.

2.3 Reverse microarrays

2.3.1 Reverse screening for the identification of tumor markers

There is substantial interest in applying proteomics to the identification of disease and tumor markers. Approaches include the comparative analysis of protein expression in normal tissue as well as in disease and cancer tissues to identify aberrantly expressed proteins. These molecules might represent new markers [93]. Protein microarrays have been used for the screening of molecular markers and pathway targets in patient-matched human tissue during disease progression. In contrast to previous pro-

tein arrays in which immobilized capture molecules are directed against certain target proteins (*e.g.* an antibody), reverse-phase protein microarrays immobilize the whole repertoire of sample proteins that represent the state of individual tissue cell populations undergoing disease transitions. Lysates are prepared from cultured cells or microdissected tissues, *e.g.* procured by LCM (laser capture microdissection), and arrayed in a microarray format in miniature dilution curves. The microarrays are screened with specific antibodies for the presence of defined target proteins. Characteristic features of the reverse microarrays include high linearity and excellent sensitivity. In addition, the sample proteins need not be labeled [94]. The reverse-phase array can use denatured lysates so that the retrieval of antigens does not pose problems. Nondenatured lysates can also be used, even when directly derived from LCM-procured tissue cells, to identify the target protein of interest as well as to elucidate protein-protein, protein-DNA and/or protein-RNA interactions. The arrayed samples in a dilution curve provide an internal standard. Direct quantitative measurements can be ascertained because the measurement lies within the linear dynamic range of the antibody-analyte interaction at any given point in the dilution curve (recently reviewed in [95]).

An alternative approach for chip-based proteome analysis is the SELDI (surface enhanced laser desorption and ionization) technology which relies on mass spectrometry as the read-out system [96, 97]. Cell extracts derived from different sources are incubated on different spots of the same adsorptive surface chemistry (*e.g.*, cation/anion exchange material, hydrophobic surfaces). After having washed away unbound proteins, the whole variety of nonspecifically captured target proteins can be analyzed by SELDI-MS. The mass spectrum shows the different molecular weights of the captured proteins. The comparison of two MS data sets generated from two different samples immediately identifies the differentially expressed proteins. In some cases, the differentially displayed proteins can be identified immediately on the basis of their molecular weights. However, it is usually necessary to enrich these proteins by affinity chromatography. This can be easily achieved by using the same adsorptive material as used for the SELDI. Enriched proteins can be identified by standard methods (*e.g.* Edman sequencing, Western blot, digest mass fingerprinting). The SELDI technology is an easy to handle tool for fast screening of differences in total protein content. As the detector sensitivity of TOF mass analyzers decreases with increasing molecular weights, SELDI is perfectly suited for the detection of small proteins and peptides. However, its sensitivity is much lower in comparison to fluorescence-labeled captured targets on antibody microarrays.

2.3.2 Cell microarrays

In the meantime, microarray technology has also been expanded to cell microarrays which can be used to study gene activity, protein expression and cell surface molecules and their binding partners. Sabatini *et al.* [98] were among the first who elaborated a microarray-based gene expression system for the functional analysis of gene products. Mammalian cells were cultured on a DNA microarray which contained the cDNAs of interest cloned into eukaryotic expression vectors. The growing cells were able to take up DNA and express the corresponding proteins. Successful transfection was monitored by the expression of green fluorescent protein which also allowed to distinguish transfected cells from untransfected cells. The living cell array is most suitable to study different functions of proteins in a cellular context [98].

Xu [99] has investigated cell microarrays in which gene activities can be determined on a phenotypic level. Bacterial or yeast cells were immobilized in a microarray format. Prior to printing, these cells were transfected with different cDNAs of interest. All cells carried a β-galactoside marker for the visualization of cell growth. By using a medium which lacked a certain compound, only those cells were able to grow which were transfected with a cDNA that could compensate for the lacking compound [99].

The printing of eukaryotic cell lines was recently described by Schwenk *et al.* [100]. Different cell lines were immobilized into a microarray format and could thus be used to characterize the binding of antibodies to cell surface proteins. This method allows the rapid screening of the specificity of cell surface protein specific antibodies.

Cells can also be used as the reporter system in a microarray-based experiment. Recently, Falsey *et al.* [101] generated microarrays which contained small molecule ligands and peptides and performed an adhesion assay with living cells. The binding specificity of the peptide against different cell lines was determined and cell function analyzed using immunofluorescence techniques. Belov *et al.* [106] developed an antibody microarray which allows the determination of more than 50 cluster of differentiation (CD) antigens on leukocytes or leukemia cells in a single experiment. A cell suspension is applied to the array; cells only bind to antibody microspots for which they express the corresponding CD antigen. Different patterns of cell binding have been obtained for normal peripheral blood leukocytes and different types of leukemia. This type of microarray can be used to perform immunophenotyping. In addition, intact cells which are captured on antibody microspots can be further characterized using soluble, fluorescently labeled antibodies.

2.3.3 Tissue microarrays

Currently, the most prominent reverse screening approach is represented by tissue microarrays. This technology was first described by Juha Kononen and Olli Kalloniemi [102] who generated a microarray of tissue samples which contained hundreds of tissue specimens. These tissue microarrays were screened for the presence or absence of DNA or RNA molecules or proteins using standard analytical methods, such as immunohistochemistry, fluorescence *in situ* hybridization (FISH), or other molecular detection methods [103]. Tissue microarrays are generated using needle biopsies that are taken from paraffin-embedded tissue specimens and embedded into a recipient paraffin block at defined array co-ordinates. The recipient paraffin block can be sectioned into a few hundred slices thereby generating hundreds of nearly identical tissue arrays. The biopsy causes minimal damage to the original tissue. Thus, it is still suitable for large-section analysis if necessary [104, 105].

One significant advantage of tissue microarrays is the large number of specimens that can be treated simultaneously in an identical manner. The conventional histological analysis of tissue specimen is a rather slow and labor-intensive process. Parallel processing of a large number of histological samples dramatically increases the throughput. However, one disadvantage of tissue microarray analysis remains. It is difficult to say whether a biopsy is representative of the whole specimen as a biopsy only represents a tiny fraction of the whole tumor. Therefore, tissue microarrays are powerful research tools when it comes to screening a large number of samples for well-defined parameters, but should be considered with caution when using them as a diagnostic tool for individual cases.

3 Conclusions

Although the principles of protein microarray technology were already described and established several years ago, its full potential has only become clear now. Useful applications for protein microarrays for the identification and quantification of proteins, as well as for protein interaction studies have been shown with different kinds of experiments. The bottleneck within the field of protein microarray technology is characterized by the limited availability of highly specific and selective capture molecules. Improvements in the generation of large sets of recombinant proteins and high-throughput generation of capture molecules might widen this bottleneck. The growing demand for alternative tools for proteome analysis supplementing the existing 2-D PAGE and mass spectrometry will certainly enhance the development of high density capture molecule arrays. Proteomic research, high-throughput drug compound screening and diagnostic applications envision a growing demand for protein microarray technologies. In medical research, protein microarrays can significantly accelerate immune diagnostics by the possibility of simultaneously analyzing all relevant diagnostic parameters. In addition, the reduction of sample volume is of great importance, in particular when the samples are limited as in the analysis of multiple tumor markers from biopsy material. Microarray technology will certainly help uncover new possibilities with respect to patient monitoring during disease treatment and therapy.

We thank Jutta Bachmann for help and critical reading of the manuscript. The work in our laboratory is supported by BMBF Grant FKZ0312879A.

Received May 8, 2003

4 References

[1] Ekins, R. P., *J. Pharm. Biomed. Anal.* 1989, *7*, 155–168.
[2] Ekins, R. P., *Clin. Chem.* 1998, *44*, 2015–2030.
[3] Finckh, P., Berger, H., Karl, J., Eichenlaub, U. *et al.*, *Proc.UK NEQAS Meeting* 1998, *3*, 155–165.
[4] Fields, S., Kohara, Y., Lockhart, D. J., *Proc. Natl. Acad. Sci. USA* 1999, *96*, 8825–8826.
[5] The Chipping Forecast, *Nat. Genet. Suppl.* 1999, *21*.
[6] Chipping Forecast II, *Nat. Genet. Suppl.* 2002, *32*.
[7] Kapranov, P., Cawley, S. E., Drenkow, J., Bekiranov, S. *et al.*, *Science* 2002, *296*, 916–919.
[8] Linck, B., Boknik, P., Eschenhagen, T., Muller, F. U. *et al.*, *Cardiovasc. Res.* 1996, *31*, 625–632.
[9] Gygi, S. P., Rochon, Y., Franza, B. R., Aebersold, R., *Mol. Cell. Biol.* 1999, *19*, 1720–1730.
[10] Anderson, N. L., Anderson, N. G., *Electrophoresis* 1998, *19*, 1853–1861.
[11] MacBeath, G., *Nat. Genet.* 2002, *32* Suppl., 526–532.
[12] Templin, M. F., Stoll, D., Schrenk, M., Traub, P. C. *et al.*, *Trends Biotechnol.* 2002, *20*, 160–166.
[13] Goldman, R. D., *Trends Biochem. Sci.* 2000, *25*, 593–595.
[14] Kausch, A. P., Owen, T. P. Jr., Narayanswami, S., Bruce, B. D., *BioTechniques* 1999, *26*, 336–343.
[15] Kohler, G., Milstein, C., *Nature* 1975, *256*, 495–497.
[16] Zhang, W. W., *Drug Discov. Today* 2003, *8*, 364–371.
[17] Valle, R. P., Jendoubi, M., *Curr. Opin. Drug Discov. Devel.* 2003, *6*, 197–203.
[18] Gao, C., Mao, S., Lo, C. H., Wirsching, P. *et al.*, *Proc. Natl. Acad. Sci. USA* 1999, *96*, 6025–6030.
[19] Knappik, A., Ge, L., Honegger, A., Pack, P. *et al.*, *J. Mol. Biol.* 2000, *296*, 57–86.
[20] Ryu, D. D., Nam, D. H., *Biotechnol. Prog.* 2000, *16*, 2–16.
[21] Krebs, B., Rauchenberger, R., Reiffert, S., Rothe, C. *et al.*, *J. Immunol. Methods* 2001, *254*, 67–84.

[22] Lecerf, J. M., Shirley, T. L., Zhu, Q., Kazantsev, A. *et al.*, *Proc. Natl. Acad. Sci. USA* 2001, *98*, 4764–4769.

[23] Raum, T., Gruber, R., Riethmuller, G., Kufer, P., *Cancer Immunol. Immunother.* 2001, *50*, 141–150.

[24] van den Brink, E. N., Turenhout, E. A., Bovenschen, N., Heijnen, B. G. *et al.*, *Blood* 2001, *97*, 966–972.

[25] Huls, G., Gestel, D., van der Linden, J., Moret, E. *et al.*, *Cancer Immunol. Immunother.* 2001, *50*, 163–171.

[26] Kreider, B. L., *Med. Res. Rev.* 2000, *20*, 212–215.

[27] Kohl, A., Binz, H. K., Forrer, P., Stumpp, M. T. *et al.*, *Proc. Natl. Acad. Sci. USA* 2003, *100*, 1700–1705.

[28] Forrer, P., Stumpp, M. T., Binz, H. K., Pluckthun, A., *FEBS Lett.* 2003, *539*, 2–6.

[29] Gunneriusson, E., Nord, K., Uhlen, M., Nygren, P., *Protein Eng.* 1999, *12*, 873–878.

[30] Gunneriusson, E., Samuelson, P., Ringdahl, J., Gronlund, H. *et al.*, *Appl. Environ. Microbiol.* 1999, *65*, 4134–4140.

[31] Brody, E. N., Willis, M. C., Smith, J. D., Jayasena, S. *et al.*, *Mol. Diagn.* 1999, *4*, 381–388.

[32] Brody, E. N., Gold, L., *J. Biotechnol.* 2000, *74*, 5–13.

[33] Hoppe-Seyler, F., Butz, K., *J. Mol. Med.* 2000, *78*, 426–430.

[34] Jayasena, S. D., *Clin. Chem.* 1999, *45*, 1628–1650.

[35] Dorrell, S., *Drug Discov. Today* 2002, *7*, 539–540.

[36] Schier, R., McCall, A., Adams, G. P., Marshall, K. W. *et al.*, *J. Mol. Biol.* 1996, *263*, 551–567.

[37] Soomets, U., Hallbrink, M., Langel, U., *Front. Biosci.* 1999, *4*, 782–786.

[38] Braasch, D. A., Corey, D. R., *Chem. Biol.* 2001, *8*, 1–7.

[39] Wilson, D. S., Keefe, A. D., Szostak, J. W., *Proc. Natl. Acad. Sci. USA* 2001, *98*, 3750–3755.

[40] Kronvall, G., Jonsson, K., *J. Mol. Recognit.* 1999, *12*, 38–44.

[41] Nord, K., Gunneriusson, E., Ringdahl, J., Stahl, S. *et al.*, *Nat. Biotechnol.* 1997, *15*, 772–777.

[42] Nord, K., Nord, O., Uhlen, M., Kelley, B. *et al.*, *Eur. J. Biochem.* 2001, *268*, 4269–4277.

[43] Dasso, J., Lee, J., Bach, H., Mage, R. G., *J. Immunol. Methods* 2002, *263*, 23–33.

[44] Carson, R. T., Vignali, D. A., *J. Immunol. Methods* 1999, *227*, 41–52.

[45] Dunbar, S. A., Vander Zee, C. A., Oliver, K. G., Karem, K. L. *et al.*, *J. Microbiol. Methods* 2003, *53*, 245–252.

[46] Joos, T. O., Stoll, D., Templin, M. F., *Curr. Opin. Chem. Biol.* 2002, *6*, 76–80.

[47] Prabhakar, U., Eirikis, E., Davis, H. M., *J. Immunol. Methods* 2002, *260*, 207–218.

[48] De Jager, W., Te Velthuis, H., Prakken, B. J., Kuis, W. *et al.*, *Clin. Diagn. Lab. Immunol.* 2003, *10*, 133–139.

[49] Chen, R., Lowe, L., Wilson, J. D., Crowther, E. *et al.*, *Clin. Chem.* 1999, *45*, 1693–1694.

[50] Fulton, R. J., McDade, R. L., Smith, P. L., Kienker, L. J. *et al.*, *Clin. Chem.* 1997, *43*, 1749–1756.

[51] Bellisario, R., Colinas, R. J., Pass, K. A., *Early Hum. Dev.* 2001, *64*, 21–25.

[52] Moody, M. D., Van Arsdell, S. W., Murphy, K. P., Orencole, S. F. *et al.*, *BioTechniques* 2001, *31*, 186–190, 192–184.

[53] Huang, R. P., *Clin. Chem. Lab. Med.* 2001, *39*, 209–214.

[54] Tam, S. W., Wiese, R., Lee, S., Gilmore, J. *et al.*, *J. Immunol. Methods* 2002, *261*, 157–165.

[55] Jenison, R., La, H., Haeberli, A., Ostroff, R. *et al.*, *Clin. Chem.* 2001, *47*, 1894–1900.

[56] Wiese, R., Belosludtsev, Y., Powdrill, T., Thompson, P. *et al.*, *Clin. Chem.* 2001, *47*, 1451–1457.

[57] Schweitzer, B., Roberts, S., Grimwade, B., Shao, W. *et al.*, *Nat. Biotechnol.* 2002, *20*, 359–365.

[58] Schweitzer, B., Wiltshire, S., Lambert, J., O'Malley, S. *et al.*, *Proc. Natl. Acad. Sci. USA* 2000, *97*, 10113–10119.

[59] Robinson, W. H., DiGennaro, C., Hueber, W., Haab, B. B. *et al.*, *Nat. Med.* 2002, *8*, 295–301.

[60] Joos, T. O., Schrenk, M., Hopfl, P., Kroger, K. *et al.*, *Electrophoresis* 2000, *21*, 2641–2650.

[61] Mezzasoma, L., Bacarese-Hamilton, T., Di Cristina, M., Rossi, R. *et al.*, *Clin. Chem.* 2002, *48*, 121–130.

[62] Bacarese-Hamilton, T., Mezzasoma, L., Ingham, C., Ardizzoni, A. *et al.*, *Clin. Chem.* 2002, *48*, 1367–1370.

[63] Harwanegg, C., Laffer, S., Hiller, R., Mueller, M. W. *et al.*, *Clin. Exp. Allergy* 2003, *33*, 7–13.

[64] Hiller, R., Laffer, S., Harwanegg, C., Huber, M. *et al.*, *FASEB J.* 2002, *16*, 414–416.

[65] Wiltshire, S., O'Malley, S., Lambert, J., Kukanskis, K. *et al.*, *Clin. Chem.* 2000, *46*, 1990–1993.

[66] Schweitzer, B., Wiltshire, S., Lambert, J., O'Malley, S. *et al.*, *Proc. Natl. Acad. Sci. USA* 2000, *97*, 10113–10119.

[67] Albala, J. S., *Expert Rev. Mol. Diagn.* 2001, *1*, 145–152.

[68] Albala, J. S., Humphery-Smith, I., *Curr. Opin. Mol. Ther.* 1999, *1*, 680–684.

[69] Emili, A. Q., Cagney, G., *Nat. Biotechnol.* 2000, *18*, 393–397.

[70] Cahill, D. J., in: Mann, M., Blackstrok, W. (Eds.), *Proteomics: A Trends Guide* 2000, pp. 47–51.

[71] Haab, B. B., Dunham, M. J., Brown, P. O., *Genome Biol.* 2001, *2*, Research 0004.0001–0004.0013.

[72] Sreekumar, A., Chinnaiyan, A. M., *BioTechniques* 2002, *Suppl.*, 46–53.

[73] Sreekumar, A., Nyati, M. K., Varambally, S., Barrette, T. R. *et al.*, *Cancer Res.* 2001, *61*, 7585–7593.

[74] Knezevic, V., Leethanakul, C., Bichsel, V. E., Worth, J. M. *et al.*, *Proteomics* 2001, *1*, 1271–1278.

[75] Scrivener, E., Barry, R., Platt, A., Calvert, R. *et al.*, *Proteomics* 2003, *3*, 122–128.

[76] Ge, H., *Nucleic Acids Res.* 2000, *28*, e3.

[77] Espejo, A., Cote, J., Bednarek, A., Richard, S. *et al.*, *Biochem. J.* 2002, *367*, 697–702.

[78] Zhu, H., Bilgin, M., Bangham, R., Hall, D. *et al.*, *Science* 2001, *293*, 2101–2105.

[79] Bulyk, M. L., Gentalen, E., Lockhart, D. J., Church, G. M., *Nat. Biotechnol.* 1999, *17*, 573–577.

[80] Zhu, H., Klemic, J. F., Chang, S., Bertone, P. *et al.*, *Nat. Genet.* 2000, *26*, 283–289.

[81] Arenkov, P., Kukhtin, A., Gemmell, A., Voloshchuk, S. *et al.*, *Anal. Biochem.* 2000, *278*, 123–131.

[82] MacBeath, G., Schreiber, S. L., *Science* 2000, *289*, 1760–1763.

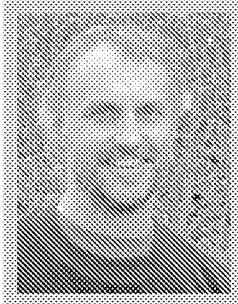
[83] Houseman, B. T., Huh, J. H., Kron, S. J., Mrksich, M., *Nat. Biotechnol.* 2002, *20*, 270–274.

[84] Lizcano, J. M., Deak, M., Morrice, N., Kieloch, A. *et al.*, *J. Biol. Chem.* 2002, *277*, 27839–27849.

[85] Reineke, U., Volkmer-Engert, R., Schneider-Mergener, J., *Curr. Opin. Biotechnol.* 2001, *12*, 59–64.

[86] Bulyk, M. L., Huang, X., Choo, Y., Church, G. M., *Proc. Natl. Acad. Sci. USA* 2001, *98*, 7158–7163.

[87] Liu, F., Iqbal, K., Grundke-Iqbal, I., Gong, C. X., *FEBS Lett.* 2002, *530*, 209–214.
[88] Michele, D. E., Barresi, R., Kanagawa, M., Saito, F. *et al.*, *Nature* 2002, *418*, 417–422.
[89] Fukui, S., Feizi, T., Galustian, C., Lawson, A. M. *et al.*, *Nat. Biotechnol.* 2002, *20*, 1011–1017.
[90] Wang, D., Liu, S., Trummer, B. J., Deng, C. *et al.*, *Nat. Biotechnol.* 2002, *20*, 275–281.
[91] MacBeath, G., Koehler, A., Schreiber, S., *J. Am. Chem. Soc.* 1999, *121*, 7967–7968.
[92] Winssinger, N., Ficarro, S., Schultz, P. G., Harris, J. L., *Proc. Natl. Acad. Sci. USA* 2002, *99*, 11139–11144.
[93] Hanash, S., *Nature* 2003, *422*, 226–232.
[94] Paweletz, C. P., Charboneau, L., Bichsel, V. E., Simone, N. L. *et al.*, *Oncogene* 2001, *20*, 1981–1989.
[95] Petricoin, E. F., Zoon, K. C., Kohn, E. C., Barrett, J. C. *et al.*, *Nat. Rev. Drug Discov.* 2002, *1*, 683–695.
[96] Davies, H. A., *J. Mol. Med.* 2000, *78*, B29.
[97] von Eggeling, F., Davies, H., Lomas, L., Fiedler, W. *et al.*, *Biotechniques* 2000, *29*, 1066–1070.
[98] Ziauddin, J., Sabatini, D. M., *Nature* 2001, *411*, 107–110.
[99] Xu, C. W., *Genome Res* 2002, *12*, 482–486.
[100] Schwenk, J. M., Stoll, D., Templin, M. F., Joos, T. O., *Biotechniques* 2002, *33*, S54–S61.
[101] Falsey, J. R., Renil, M., Park, S., Li, S. *et al.*, *Bioconjug. Chem.* 2001, *12*, 346–353.
[102] Kononen, J., Bubendorf, L., Kallioniemi, A., Barlund, M. *et al.*, *Nat. Med.* 1998, *4*, 844–847.
[103] Kallioniemi, O. P., Wagner, U., Kononen, J., Sauter, G., *Hum. Mol. Genet.* 2001, *10*, 657–662.
[104] Mousses, S., Kallioniemi, A., Kauraniemi, P., Elkahloun, A. *et al.*, *Curr. Opin. Chem. Biol.* 2002, *6*, 97–101.
[105] Simon, R., Sauter, G., *Exp. Hematol.* 2002, *30*, 1365–1372.
[106] Belov, L., de la Vega, O., *Cancer Res.* 2001, *61*, 4483–4489.



Dr. Thomas Joos is head of the Biochemistry Department of the NMI Natural and Medical Sciences Institute at the University of Tuebingen. The NMI is a research foundation supported by the Ministry of Commerce of the Baden-Württemberg state. The Institute is associated with the University of Tuebingen. Scientists in the areas of applied and theoretical physics, chemistry, physical chemistry, biology and biochemistry are experienced in system analysis and problem solution for industrial clients. The R + D activities at the NMI are characterized by their multidisciplinary approach. Dr. Joos has been with the NMI since 1998, where he has been responsible for protein microarray technology. Prior to joining the NMI, Dr. Joos did his postdoctoral research in the laboratory of Prof. Peter Hausen at the Max-Planck-Institute of Developmental Biology, Department of Cell Biology, researching cell-cell and cell-matrix interaction during early embryogenesis of *Xenopus laevis*. Dr. Joos studied Biochemistry at the University of Tuebingen. He received his Ph.D. degree in 1985 on integrin-α_5 during early embroygenesis of *Xenopus laevis*.